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Review

Mitochondrial metabolism and aging in the filamentous fungus *Podospora anserina*

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Abstract

The filamentous fungus *Podospora anserina* has a limited lifespan. In this organism, aging is systematically associated to mitochondrial DNA instability. We recently provided evidence that the respiratory function is a key determinant of its lifespan. Loss of function of the cytochrome pathway leads to the compensatory induction of an alternative oxidase, to a decreased production of reactive oxygen species and to a striking increase in lifespan. These changes are associated to the stabilization of the mitochondrial DNA. Here we review and discuss the links between these different parameters and their implication in the control of lifespan. Since we demonstrated the central role of mitochondrial metabolism in aging, the same relationship has been evidenced in several model systems from yeast to mice, confirming the usefulness of simple organisms as *P. anserina* for studying lifespan regulation.

Keywords: Mitochondrion; Aging; Respiratory chain; Alternative oxidase; ROS; Mitochondrial DNA stability; *Podospora anserina*

1. Introduction

Numerous studies have dissected the role of mitochondria in aging in different organisms ranging from unicellular eukaryotes to mammals and provided support for a central role of mitochondrial metabolism in the control of lifespan.

A complex network of intricate processes is controlling aging leading to different mechanisms and rates of aging depending on the organ, tissue and cell type studied. Under these conditions, the use of simple organisms may shed light on some of these mechanisms. It has long been believed that organisms without clear soma-germline distinction do not age. However, the budding yeast *Saccharomyces cerevisiae* became an accepted model in aging research during the 1980s and allowed to map important functions that regulate the pace of aging from yeast to metazoans.

The first model in which the role of mitochondria in aging has been established is the filamentous fungus *Podospora anserina*. In this organism, aging research started in the early 1950s when G. Rizet described that all the cultures of this filamentous ascomycete present an unavoidable arrest of vegetative growth, that he called senescence [1,2].

In *P. anserina*, senescence is maternally inherited and early studies revealed the presence of a cytoplasmic and infectious factor that accumulates and triggers senescence [3] reviewed in [4,5]. Molecular analysis revealed that the senescent state is systematically associated with alterations of the mitochondrial DNA (mtDNA): as the culture ages, mutated mtDNA molecules harboring particular deletions and rearrangements accumulate, eventually leading to loss of wild-type mtDNA. Although these mitochondrial DNA modifications have been proposed to correspond to the « senescence factor », the point is far from clear and the nature of this factor is still puzzling at the present time. In contrast, the key role of the respiratory function in the control of longevity was first demonstrated non-equivocally in *P. anserina*. It is quite interesting to note that since this demonstration such a link between respiratory metabolism and lifespan has also been reported in a large spectrum of model systems from yeast to mice.

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Previous reviews on the senescence process in *P. anserina* and aging in other filamentous fungi are available [4–7]. In this paper, we intend to summarize recent data obtained in *P. anserina* concerning the link between respiration and longevity. We describe the properties of mutants deficient for the "normal" respiratory cytochrome pathway (see Fig. 2), discuss the role of the alternative oxidase, of reactive oxygen species and ATP generation in the control of lifespan and emphasize the critical importance of the respiratory function in the aging process of numerous species.

1.1. The senescence phenomenon

As mentioned in Introduction, *P. anserina* has a limited vegetative growth and after several divisions, apical cells stop growing and die. The cessation of growth is accompanied with a dark pigmentation (Fig. 1) attributable to the accumulation of lipofuscin [8]. As senescence proceeds, the mitochondrial genome is destabilized: there is an accumulation of multiple mtDNA rearrangements and deletions. This accumulation is paralleled by the elimination of the wild-type mtDNA [9,10]. It is very likely that the disappearance of the wild-type mtDNA is the actual cause of the death in this obligate aerobic organism, meaning that the control of the mtDNA stability is closely linked to longevity in *P. anserina*. One of the altered mtDNA molecules (called senDNAα or plDNA) accumulate in all senescent wild-type strains. It corresponds to multimers of the first intron of the *cox1* gene. Because of its systematic accumulation in senescent cultures, the senDNAα has been thought for a long time to have a prominent role in the senescence process of *P. anserina*. However, this idea has been questioned by the analysis of some long-lived mutants that senesce without accumulation of senDNAα (or plDNA) [11,12] and it has been definitively refuted by the selection of a mutant devoid of intron α which nevertheless displays a systematic senescence process. In this strain, senescence is associated with the accumulation of a variety of other mtDNA rearrangements [13]. This demonstrates that the senDNAα is generated by a "private" non-causal mechanism, accompanying aging in *P. anserina*. However, aging seems to be systematically correlated with mtDNA instability in this organism.

1.2. Loss of function of the cytochrome respiratory pathway results in a spectacular increase of longevity in P. anserina

Genetic experiments have revealed that longevity is controlled by nuclear and mitochondrial traits. Mitochondrial mutants with an abnormally long lifespan have been selected: these mutants are potentially escaping senescence. Interestingly, most of them carry a deletion of the mtDNA covering a part of the intron α as well as a part of the first exon of the *cox1* gene [14–16]. As a result, these mutants cannot accumulate senDNAα and are deficient for cytochrome *c* oxidase activity. As a matter of fact, absence of the cytochrome respiratory pathway is not lethal in the strict aerobe *P. anserina* thanks to the presence of an alternative oxidase (AOX) [17,18]. A schematic diagram of the flow of electrons through the respiratory chain of *P. anserina* is presented in Fig. 2. In order to directly test the effects on longevity of a complete absence of cytochrome *c* oxidase (complex IV), a mutant disrupted for the nuclear gene *cox5* (encoding subunit V of cytochrome *c* oxidase) was constructed [17]. This *cox5*::*ble* mutant displays a severe alteration in germinating mycelium, a thin and poorly colored growing mycelium, female sterility and a 50% reduction of its growth rate. However the most spectacular effect of the mutation is the resulting increase of lifespan: whereas lifespan of wild-type cultures is about 25 days in laboratory conditions at 27 °C, it is more than 2 years for most of the *cox5*::*ble* cultures, which means an increase of lifespan of 3000%. Interestingly, no rearrangement of mitochondrial DNA was observed in *cox5*::*ble* cultures during growth. Another striking feature of this mutant and of the mitochondrial mutants deleted for *cox1* gene is the 2- to 3-fold decrease of reactive oxygen species (ROS) production.

Fig. 1. Juvenile growing wild-type culture (left) and senescent wild-type culture (right).

It is worth noting that a loss of function of complex III also leads to a considerably increase of lifespan, decreased ROS production and reduced mitochondrial DNA alterations (Sellem, C., personal communication), demonstrating that these effects are not specific to the absence of complex IV but are due to the absence of a functional cytochrome respiratory pathway.

These results provide direct evidence of a causal link between respiration and longevity and clearly demonstrate that the cytochrome respiratory function is a key process in shortening lifespan and destabilizing the mitochondrial genome.

1.3. Possible ways by which respiration controls lifespan and mitochondrial DNA stability

In loss of function mutants for the cytochrome pathway, respiration proceeds completely via the AOX-dependent pathway. An important feature of the alternative oxidase is that it does not couple the electron transfer to proton translocation. Because this pathway branches off at the ubiquinone pool, its contribution to energy production is approximately one third that of the cytochrome pathway (Fig. 2). The slow growth rate, female sterility, alteration of the germination capacities and mycelium morphology of the cytochrome pathway mutants are generally attributed to a reduced ATP production due to the exclusive use of the alternative pathway. Another property of this pathway is to divert electrons from oxygen when the cytochrome pathway is blocked, thus limiting the production of mitochondrial ROS under these conditions. Several studies have reported an inverse relationship between the abundance of AOXp and H_2O_2 in plant cells [19–21]. It is thus highly probable that the reduction of ROS production in the long-lived cytochrome pathway mutants of *P. anserina* results from the ability for the electron flux to resume through this pathway.

1.3.1. Links between longevity and expression of the alternative oxidase

In *P. anserina*, the alternative pathway is not active under normal conditions. In contrast, it is strongly induced in all the respiratory mutants studied that correspond to a complete or a partial loss of the cytochrome pathway [18,22–24]. A correlation between the AOX protein level and lifespan extension has been reported in different mutants [18,23]. However, it seems that this correlation is neither systematic, nor causal.

Indeed, in order to directly test the implication of the alternative oxidase in the control of *P. anserina* longevity, strains inactivated for the *aox* gene or strains overexpressing constitutively the AOX protein have been constructed [22]. Constitutive expression of the AOX protein and its inactivation has no impact on the phenotype and longevity of strains possessing a functional cytochrome pathway. Of course, inactivation of the *aox* gene in the *cox5*::*ble* mutant leads to lethality. Surprisingly, the constitutive overexpression of AOXp in the *cox5*::*ble* mutant leads to a spectacular decrease of lifespan and to the restoration of a senescence process accompanied with mitochondrial DNA instability. This clearly dissociates the increase of lifespan from the level of AOXp in cytochrome-deficient mutants.

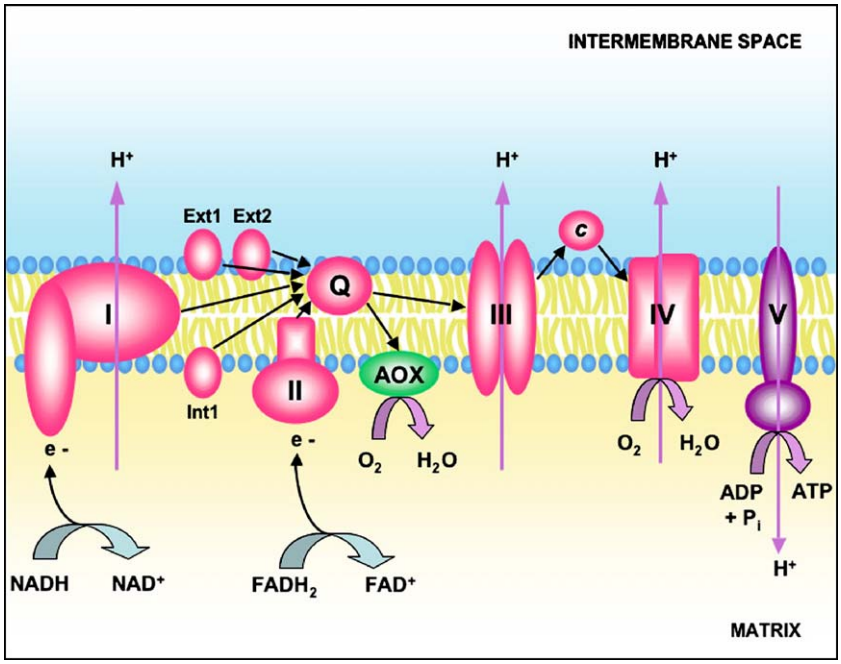

Fig. 2. Schematic representation of the hypothetic respiratory chain of *Podospora anserina*. Complex I: NADH dehydrogenase, complex II: succinate dehydrogenase, complex III: cytochrome *bc1*, complex IV: cytochrome *c* oxidase, complex V: ATPase, Q: ubiquinone pool, AOX: alternative oxidase, *c*: cytochrome *c*, Ext1 and Ext2: external NAD(P)H dehydrogenases, IntI: internal NAD(P)H dehydrogenase. The cytochrome pathway utilizes complexes I, II, III and IV. The electron transfer activity of complexes I, III and IV is used to pump protons across the inner mitochondrial membrane, from the matrix into the intermembrane space. The resulting proton gradient drives the synthesis of ATP by complex V. If electron flow through the cytochrome pathway is compromised, it is diverted towards the alternative oxidase branched at the level of ubiquinone. When the alternative oxidase is exclusively used, electron flow and proton pumping are coupled only for electrons entering by complex I; two of the three energy coupling sites are wasted.

Consistent with this observation are the data obtained by Sellem et al. [24]: in a mutant (Δ*oxa1 (oxa1^{ts}) rmp1-2*) partially deficient in complexes I and IV activities, lifespan is considerably extended and the alternative oxidase is strongly induced. However, the inactivation of the *aox* gene in this mutant does not significantly decrease lifespan. This result and the previous observation demonstrate that there is no causal relationship between the extension of lifespan and the presence of AOX in long-lived cytochrome-impaired mutants.

1.3.2. Links between longevity, mitochondrial DNA stability, ROS production and metabolic rate

The free radical theory of aging states that aging is due to the progressive accumulation of ROS-inflicted damage, including mtDNA mutations, the accumulation of which has been postulated to lead to a "vicious cycle" of further mitochondrial ROS generation and mitochondrial dysfunction [25]. Several lines of evidence, specially correlation between increased lifespan and enhanced resistance to oxidative stress in several mutants of *C. elegans* and *Drosophila* support this hypothesis [26–32]. Today, ROS generation remains the most widely accepted cause of aging. However, several data conflict with this hypothesis [33,34] and recent studies of mutant mice accumulating high levels of mtDNA mutations show that premature aging in these animals is not associated with an increase of oxidative stress [35,36].

A large body of observations also indicates that there is a link between metabolic rate and longevity. Perhaps, the most straightforward relationship between these two parameters is the observation that dietary restriction extends lifespan in many organisms including *C. elegans*, *Drosophila* and rodents. Similarly, reduction of available glucose in the medium, also leads to longer life in *S. cerevisiae* [37] and *P. anserina* [38]. However the nature of this relationship remains unclear [33]. The initial hypothesis for a constant energy potential [39] has been discredited. It has developed into a "metabolic/oxidative theory" which stipules that a slowing of the rate of living is linked to a reduction of energy consumption, to a reduction of ROS and molecular injuries leading to an increase of lifespan [26,40,41]. It is worth noting that many long-lived slow-living mutants do not support this direct link between longevity and metabolism. For example, since the slow living *clk-1* mutant of *C. elegans* has normal metabolism and ATP levels, extension of its lifespan cannot be attributed to a reduced ATP production or to increased antioxidant enzymes levels [42,43]. In the same way, it seems that caloric restriction does not work simply by reducing metabolic rate but corresponds to a highly regulated process [44].

Metabolic activity of the long-lived cytochrome-deficient mutants of *P. anserina* has not been determined exhaustively. However, we and others showed that the production of ROS is decreased in loss of function mutants [17] and that oxygen uptake is significantly higher than in the wild-type [23] (Sellem, C., unpublished results). Although measures of ATP content have not been performed, it seems reasonable to correlate the "slow-living" phenotype and the sterility of these mutants to a decrease in ATP production resulting from the loss of two potential coupling sites for proton transport. These data thus agree with the numerous observations of an inverse relationship between metabolic rate and longevity. The suppressive effect of the constitutive overexpression of AOXp in cytochrome-deficient mutants provides a supplementary result in agreement with an inverse correlation between respiratory metabolism and lifespan. In cytochrome-deficient mutants over-expressing AOXp, growth rate and fertility are improved. This improvement is associated with restoration of wild-type levels of ROS, with mitochondrial DNA instability and with senescence. It may seem paradoxal that overexpression of AOXp leads to an increased ROS level in cytochrome-deficient mutants since AOXp is expected to prevent overreduction of upstream electron transport components favoring ROS formation. Indeed, the decrease of ROS level observed in cytochrome-deficient mutants is explained by this property: the exclusive use of AOXp probably reduces the ATP/ADP ratio and then membrane potential and ROS level. However as we have proposed [22], overexpression of AOXp in these mutants should lead to an increased electron flow through the alternative pathway (more abundant). This increase would be accompanied with an increased oxygen consumption and an increased ATP formation at the first coupling site. A higher ATP level (higher ATP/ADP ratio) is expected to lead to an increased membrane potential restoring normal ROS formation.

If this interpretation is correct, it supports the "metabolic/oxidative" theory: higher entry of electrons through complex I generates higher levels of ATP and ROS and a decreased lifespan.

There are many sources of mitochondrial DNA damage. Among the intrinsic sources are the ROS and it is generally assumed that the high mutation rate of mitochondrial DNA is due to its chronic exposure to mitochondrial ROS. Mutations of mitochondrial DNA have been shown to accumulate with aging in several tissues of various species [45–47]. However a causative link between these mutations and aging has only been established recently in mice expressing an error-prone mitochondrial DNA polymerase [35,48]. In wild-type cultures of *P. anserina*, a systematic correlation between aging and mitochondrial DNA rearrangements has been described some 20 years ago. In mutants whose cytochrome pathway is compromised, decreased ROS production is always accompanied with mitochondrial DNA stability and increased lifespan; any restoration of wild-type levels of ROS in these mutants is accompanied with mitochondrial DNA instability and decreased lifespan [22,24]. These data suggest that ROS are implicated in the production and/or the accumulation of the mitochondrial DNA rearrangements observed during aging in *P. anserina*. This implication could be direct in generating oxidative lesions to DNA, or it could be indirect by oxidation of proteins required for DNA replication and/or maintenance. However, the specificity and the systematic occurrence of these rearrangements during senescence of the wild-type strain (accumulation of senDNAα corresponding exactly to the first intron of the mitochondrial *cox1* gene) remains a mystery.

In summary, the data obtained by manipulating the respiratory metabolism in *P. anserina* indicate a systematic link between efficiency of respiratory metabolism, ROS level, stability of the mitochondrial DNA and longevity. On the

contrary, the AOXp level can clearly be dissociated from the stability of the mitochondrial DNA and from longevity. The occurrence of an alternative pathway in *P. anserina* is however significant. It may slow down generation of ROS when the electron flow through the cytochrome chain is compromised. This would explain, in the frame of the free radical theory, why dysfunction of complexes III or IV results in decreased ROS level and increased lifespan in *P. anserina* whereas in animals devoid of an alternative oxidase such as *C. elegans*, it can result in increased ROS level and decreased lifespan [49,50]. The situation of the *P. anserina* respiratory mutants is reminiscent to that of long-lived mice that display a higher oxygen consumption associated with an increased degree of respiratory uncoupling [51].

An alternative hypothesis concerning the control of longevity by the ROS levels can be put forward. It is well known that ROS have additional functions besides those related to oxidative stress. These involve the use of ROS as second messengers in events required for cell growth and differentiation. The regulation of nuclear gene expression by the functional state of the mitochondria and/or by ROS has been described in several systems and signaling from mitochondria to nucleus called retrograde regulation is involved in the control of longevity in yeast [52–54].

1.4. Respiration and aging in other system models

In conclusion, we would like to point out that since the 1980s, when the implication of mitochondria in aging has been clearly demonstrated in *P. anserina*, a number of studies have demonstrated the involvement of the mitochondrial metabolism in the aging process in a broad diversity of organisms. In *C. elegans*, mutation in a component of complex III considerably increases lifespan [55]. In the same way, lifespan is increased by RNAi inhibition of respiratory chain components of complexes I, III, IV and V at early stage of development [56]. Similarly, a systematic RNAi screen revealed that a great number of lifespan determining genes are related to mitochondria [57]. In *S. cerevisiae*, lifespan is controlled by respiration: in low glucose media, increased electron transport and respiration rate, lead to an extension of lifespan [37,58]. In *Drosophila*, a role for mitochondrial energy metabolism in aging is suggested by the *Indy* mutation that inactivates a dicarboxylate co-transporter and increases lifespan [59]. Numerous studies in humans and mammals have demonstrated a correlation between aging and respiratory chain deficiencies [47]. In humans, primates and rodents, mitochondrial mutations have also been reported to accumulate in a variety of tissues during aging [60–62].

The causative effect of these mitochondrial mutations in the process of aging has been intensively debated. It has been recently demonstrated in mice expressing a proofreading deficient version of the mitochondrial DNA polymerase [35,48]. These mice exhibited higher mitochondrial DNA instability and a shortened lifespan. The mechanism(s) leading to premature aging through mitochondrial DNA mutations are still obscure. According to the "vicious cycle" theory, mtDNA mutations would contribute to increase ROS production and oxidative stress. Recent studies rather support that mtDNA mutations control longevity by promoting apoptosis [35]. These results strongly support the view that accumulation of mtDNA mutations plays a key role in the aging process, they show that this accumulation is not accompanied with nor due to an increased ROS production in mutator mutant mice. However, they do not mean that during "normal" aging, generation of ROS plays no role in generation and accumulation of mtDNA mutations.

These whole data clearly show the relevance of research on simple organisms for deciphering complex multi-factorial processes as aging. The study of model systems from yeast to worms has shown that conserved pathways govern the pace of aging in all eukaryotes. The validity and power of *S. cerevisiae* as a model for aging has been truly established. It has led to the identification of genes and pathways controlling chromatin structure, genome stability and metabolism, whose some counterparts are also involved in animal aging. In the same way, *P. anserina* that is a multicellular, strict aerobe possessing an alternative pathway of respiration able to bypass the usual respiratory chain, appears particularly relevant for the study of mitochondrial involvement in aging. We are convinced that future studies on this model system will improve our understanding of the intricate relation(s) between mitochondrial DNA stability, ROS level and aging.

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